Iranian Journal of Clinical Infectious Diseases
2008;3(4):179-183

ORIGINAL ARTICLE

Production of mutant streptokinase recombinant protein

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ABSTRACT

Background: Streptokinase (SK) is most widely used for treatment of myocardial infarction, however, it is the most expensive thrombolytic agent. A major drawback to SK use is the widespread presence of anti–streptokinase antibodies (Abs). These Abs cause allergic reactions and neutralize streptokinase therapeutic effects.

Materials and methods: To produce an engineered variant of streptokinase being functional and less antigenic than the native molecule, we cloned and expressed streptokinase mutant gene lacking the C – terminal 42 amino acids. Recombinant protein was confirmed by western blot analysis with anti T7 monoclonal antibodies.

Results: pGEMEX-1 expression vector contains T7 gene 10 protein as fusion protein immediately down stream of T7 promoter and before multiple cloning site, streptokinase mutant gene was cloned after fusion protein.

Conclusion: We cloned and expressed mutant streptokinase gene, lacking the C-terminal 42 amino acids. If mut-C42 activity was less affected by neutralizing antibodies compared with native streptokinase, this engineered variant could be a preferred alternative to native streptokinase for thrombolytic therapy.

Keywords: *Streptokinase, Myocardial infarction. Mutation, Cloning.*
(Iranian Journal of Clinical Infectious Diseases 2008;3(4):179-183).

INTRODUCTION

A blood clot developed in circulatory system can cause vascular blockage leading to death. In mammalian, the enzyme responsible for fibrinolysis is plasmin. The active plasmin is produced from the inactive plasminogen which is mediated by the various plasminogen activators. Streptokinase, a protein consist of 414 amino acids residues produced by β hemolytic streptococci has been used in the therapy of acute myocardial infarction (AMI) for its strong activity in dissolving fibrin. This protein is a plasminogen activator (1). Shortly after discovering the fibrinolytic effects of streptokinase, its immunogenicity was reported (2). Antibodies to streptokinase are found in most individuals due to the high frequency of streptococcal infections (3). Moreover, when patient receives streptokinase, antibody titers raise. These neutralizing antibodies reduce the efficiency of thrombolytic therapy and may cause a range of allergic reactions (4-6).

Previous reports have identified 5 antigenic regions in SK localized to fragments spanning amino acid residues 1-13, 14-127, 1-253, 120-353, 353-414 (7,8). Some studies are performed based on evidences about the presence of an immunodominant epitope in the C-terminal region of native molecule (9,10). A deletion mutant (mut-C42), lacking the C-terminal 42 amino acids,

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showed to be less recognized and less neutralized by anti-streptokinase antibodies from patients sera respect to the native protein (11,12).

Comparative clinical trials in the treatment of acute myocardial infarction suggested that streptokinase is a cost effective and useful thrombolytic drug (1). Hence, several studies have been conducted focusing on production and improvement of streptokinase (13-16).

To compare the reactivity of anti streptokinase antibodies to mut-C42 and native streptokinase in Iranian patients' sera, we cloned and expressed the streptokinase mutant gene lacking the C–terminal 42 amino acids in pGEMEX-1 expression vector for further studies.

MATERIALS and METHODS

We used cloned streptokinase mutant gene which have been cloned in pGEMEX-1 expression vector at SacI and BamHI restriction sites during another our study (16). Competent cells were prepared by E.coli XL1-Blue based on Hnanahan method (17) and transformed with recombinant pGEMEX-1 plasmid. A bacterial colony contained recombinant plasmid was mass cultured in LB medium and plasmid was extracted (18). Streptokinase mutant gene (1116bp) was amplified by PCR (19). PCR mix contained 1x PCR buffer, 0.1mM dNTP, 1.5mM MgCl2, 100ng recombinant pGEMEX-1 plasmid DNA, 40pmol of each forward and reverse primers and 1.25unit Taq DNA polymerase in 30µl volume.

PCR reaction was achieved by flowing parameters: denaturation at 94ºC for 30 seconds, annealing at 60ºC for 1 minute and extension at 72ºC for 40 seconds, repeated 30 cycles. PCR product was electrophoresed on 1% low melting point agarose gel and DNA band was sliced under long wave UV and recovered by DNA extraction kit (Fermentas Cat. No k0513). PCR product was sequenced and deposited to Gene Bank.

Gene expression from the pGEMEX-1 Vectors is based on the T7 expression system which uses a convenient vector/host combination for high-level expression of cloned genes in vivo. Sequences cloned into the pGEMEX1- Vectors are expressed as T7 gene 10 fusion proteins in JM109 (DE3) or BL21 (DE3) pLysS host strains containing an inducible gene for T7 RNA polymerase. JM109 (DE3) is a specially constructed host strain that contains an IPTG inducible gene for T7 RNA polymerase. PGEMEX -1 plasmid was transformed into the bacterial strain JM109 (DE3) (Promega, technical bulletin no.2, www.promega.com). A colony contained recombinant plasmid was cultured on shacking incubator for overnight at 37°C in 50ml LB medium containing 100µg/ml ampicillin. The next day, 1–2ml of culture was removed and inoculated into 50ml fresh LB medium containing 100µg/ml ampicillin. The culture was grown up to an A600 of 0.6. Then induced with 1mM IPTG (isopropyl betathio galactopyranoside) and incubated the culture for an additional 3–5 hours. For sampling, we removed 100–200µl of the culture and cells were precipitated by micro centrifuge. The bacterial pellet was resuspended in 100µl of resuspension buffer (100 mM Tris–HCl pH 8, 20% glycerol, 4% SDS, 2% β-mercaptoethanol, 0.2% bromophenol blue) to solubilize the fusion protein. Sample was centrifuged to pellet the insoluble fusion protein. The sample in parallel with uninduced control culture was analyzed by SDS-PAGE. The gel was stained with Coomassie brilliant blue R-250 (20).

For western blot analysis, SDS-PAGE gel was transferred to nitrocellulose membrane and analyzed by western blot. Briefly, nitrocellulose sheet was immersed in 3% bovine serum albumin in Tris buffered saline, 0.1% Tween 20 at room temperature for 1 hour to block excess protein-binding sites. The nitrocellulose sheet was reacted with T7 mono clonal antibody as primary antibody at room temperature and washed in TBS buffer.

Immune reactions were identified by secondary antibody (anti sheep anti mouse IgG conjugated to horseradish peroxidase). Color development (antigen–antibody reaction) was observed by addition of Di Amino Benzidine (DAB)/H2O2 as substrate (21,22).

RESULTS

Expression vector: pGEMEX-1 expression vector contain T7 gene 10 protein (figure 1) as fusion protein immediately down stream of T7 promoter and before multiple cloning site, streptokinase mutant gene was cloned after fusion protein (figure 2). Expressed gene will be fused with T7 gene 10 protein and will be detected by T7 monoclonal antibody by western blot analysis.

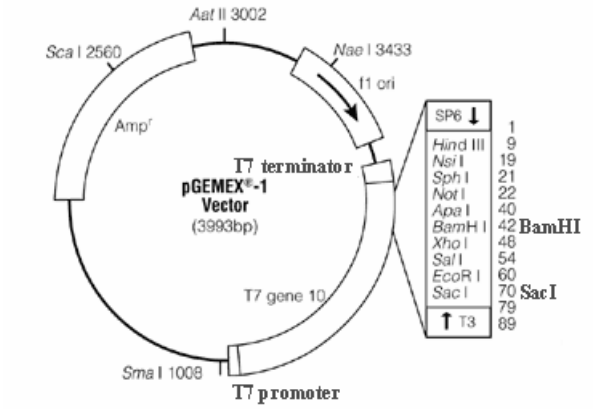

Figure 1. Map of pGEMEX-1 expression vector

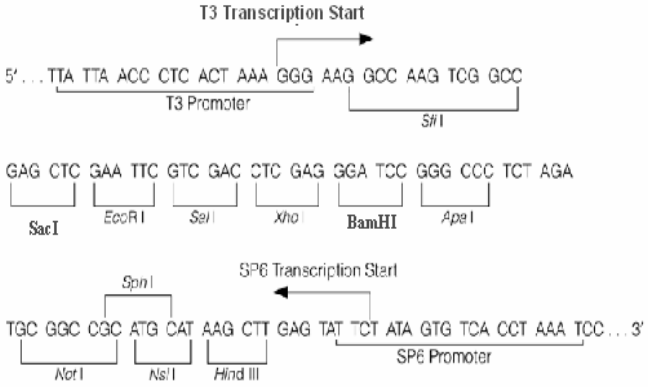

Figure 2. Multiple cloning site of pGEMEX-1 expression vector

PCR reaction: Figure 3 shows 1% agarose gel electrophoresis of 1116 bp as PCR product of streptokinase mutant gene. PCR product was sequenced and deposited to Gene Bank at Accession No. EF012822.

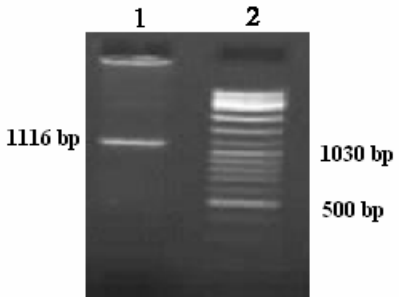

Figure 3. 1% agarose gel electrophoresis. *Lane 1*: Streptokinase mutant PCR product. *Lane 2*:100bp DNA ladder marker.

SDS-PAGE: Collected bacterial samples were lysed by lysis buffer and subjected to electrophoresis on 10% SDS-PAGE and stained by coomassie brilliant blue G250. Figure 4 shows the stained SDS-PAGE and expressed recombinant protein is shown in lane 4 (74 KDa: 42 KDa for streptokinase and 32 KDa for T7 gene 10 protein) at 5 hour after induction by IPTG.

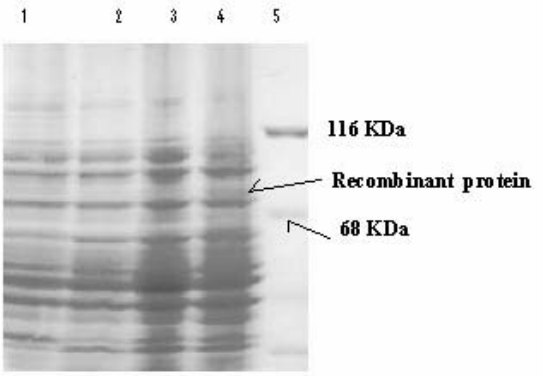

Figure 4. 10% SDS-PAGE. *Lane 1*: Lysate cells of JM109. *Lane 2*: Lysate bacterial cell containing pGEMEX-1. *Lane 3*: Lysate of JM109 cells containing rpGEMEX-1 before induction. *Lane 4*: Lysate of JM109 cells containing rpGEMEX-1 5 hours after induction. *Lane 5*: Protein size marker.

Western blotting: SDS-PAGE gel was transferred on nitrocellulose membrane and detected by colorimetric method (western blot serological test).

DISCUSSION

Several different M serotypes of group A streptococci can cause human plasma to clot in nutrient-poor media (23). A blood clot developed in circulatory system can cause vascular blockage leading to death and circulatory plasmin enzyme is responsible for fibrinolysis in mammalian. The active plasmin is produced from the inactive plasminogen which is mediated by the various plasminogen activators. Streptokinase is a plasminogen activator and has been used in the therapy of acute myocardial infarction for its fibrin dissolving activity (1). Plasminogen activators are agents that are currently applied as thrombolytic therapy to achieve rapid vascular reperfusion (24). Previous reports have shown 5 antigenic regions in streptokinase, mapped with soluble recombinant streptokinase fragments and anti-SK Abs from human sera from patients treated with streptokinase. These regions comprise amino acids 1-13, 1-253, 120-352 (containing two distinct, non overlapping epitopes) and 353-414. The data indicated that all patients raise antibodies against two major discrete regions, 1-253, 120-352. Two other epitopes in streptokinase constructed by amino acids 1-13 and 353-414 were not antigenic in all humans tested in this study (8). Torrens et al mapped the antigenic regions (linear epitopes) located on streptokinase, using human total sera from patients treated with Heberkinase. As a result they showed that C-terminal region 380-490 among other fragments is notably immunodominant (9). Ojalvo et al reported that 30% of 1008 normal donors recognized a synthetic peptide resembling amino acids 373-414 from streptokinase C-terminal region (10). Then immunoreactivity of the synthetic 42–residue peptide with sera from AMI patients before and after Heberkinase therapy was tested. This peptide was recognized by 39% of patients before therapy and as it was expected, recognition increased to 64 % after therapy. This is evidence about immune response towards C-terminal region of streptokinase (9,12). These studies were complemented by performing a competition experiment in which both native and mutant (lacking C-terminal 42 amino acids) proteins were tested in a neutralizing activity assay using patient's sera. For most of the individuals, mut-C42 neutralizing activity titer (NAT) significantly decreased with respect to native SK-NAT and mut–C42 was significantly less recognized by pre-existing anti-streptokinase antibodies than the native streptokinase (9). Then, the immune response to mut-C42 and native molecule was tested in animals. 14 monkeys were subjected with native molecule and mut-C42. All monkeys developed anti-streptokinase antibodies, but in the case where treatment induced antibodies directed against the C-terminus of SK, neutralizing activity against the native protein was significantly higher than that developed against mutant-C42 (11). This data indicate that the immunodominant epitope in the C-terminal of native streptokinase can be eliminated without loss of specific activity.

We cloned and expressed mutant streptokinase gene, lacking the C-terminal 42 amino acids. If mut-C42 activity was less affected by neutralizing antibodies compared with native streptokinase, this engineered variant could be a preferred alternative to native streptokinase for thrombolytic therapy.

ACKNOWLEDGEMENT

This study was supported by vice chancellor of research affair of Shahid Beheshti University, M.C.,(project No, 9852) and was done in Cellular and Molecular Biology Research Center. The authors thank directors and all staff of the laboratory.

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